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Electronic Supplementary Information (ESI)

Demonstration of Cellular Imaging by Using Luminescent and Anti-Cytotoxic Europium-doped Hafnia Nanocrystals

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EXPERIMENTAL METHODS

1. Synthesis and characterization of Eu^{3+} doped hafnia nanocrystals.

Chemicals. Hafnium(IV) tert-butoxide (99.9 %, Strem Chemicals), anhydrous benzyl alcohol (99.8 %, Aldrich), europium acetate hydrate (99.99 %, ABCR), diethyl ether (99.5 %, Fischer Chemicals), ethanol (absolute, Fischer Chemicals) and 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (technical grade, Aldrich), were purchased and used without further purification.

NCs synthesis. Eu:HfO_2 nanocrystals were synthesized by the benzyl alcohol route inside a glovebox (Ar, O_2 and H_2O < 0.1 ppm), according to previous work.[29] Briefly, 0.072 mmol of europium acetate was added to anhydrous benzyl alcohol (BnOH) into a glass test tube, followed by the addition of hafnium(IV) tert-butoxide to the mixture. A total amount of 2.4 mmol of precursor and a total volume of 20 mL mixtures were used, leading to a nominal Eu^{3+} concentration of 3 %mol. The reaction mixture was heated at 220 °C in a steel autoclave (acid digestion vessel, Anton Parr, USA) for 96 h. After synthesis, the precipitate was collected by centrifugation and washed with diethyl ether. The wet product was suspended in 10 ml ethanol and 300 µL of 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (MEEAA) was added leading to a clear dispersion. The nanocrystals were precipitated with ether and redispersed in ethanol twice to remove excess ligand. After drying overnight at 60 °C, the nanocrystals were redispersed in water with a concentration up to 50 mg/ml.

Structural characterization. X-ray diffraction (XRD) measurements were performed on functionalized dry powdered nancrystals in reflection mode (Cu Ka radiation at 45 kV and 40 mA) on an Empyrean diffractometer from PANalytical (The Netherlands). Transmission electron microscopy (TEM) and High-resolution transmission electron microscopy (HRTEM) in both, TEM and scanning (STEM) modes was performed on a FEI Talos F 200X operated at 200 kV. STEM analyses were carried out with a high angle annular dark field detector (HAADF STEM) and were accompanied by the high resolution energy dispersive spectroscopy (EDS) using the SuperX integrated EDS-system with four silicon drift detectors (SDDs). The EDS-STEM analyses were performed with a probe size of 0.5 nm. The samples were prepared by dropping 10 µl of water dispersion of Eu:HfO_2 NCs (1 mg/ml) on graphene-enhanced lacey carbon Ni grids from Electron Microscopy Sciences.

Photophysical Studies: Steady state PL and PLE spectra were measured by a xenon lamp as excitation source, together with a double monochromator (Jobin-Yvon Gemini 180 with a 1200 grooves/mm grating), and recorded through a nitrogen cooled CCD detector coupled to a monochromator (Jobin-Yvon Micro HR). Under CW laser excitation at 532 nm signals were recorded by a nitrogen cooled CCD coupled with a double monochromator Triax-190 (Horiba Jobin-Yvon), with a spectral resolution of 0.5 nm. The recorded spectra were corrected for the setup optical response. The details of the QY measurements are reported as Supporting Information. For time-resolved PL measurements we used a Continuum Minilite nanosecond pulsed laser (5 ns pulse width). Time-resolved PL spectra have been detected in photon-counting mode using a Edimburgh L900 Flash Photolysis setup, with a temporal resolution <1 ns. PL and time resolved PL on stained C2C12 cells were acquired with a Nikon C1 inverted confocal microscope coupled with the 532 nm laser and to an ORTEC digital multichannel scaler, with a temporal resolution of 0.1 ns.

Radioluminescence Studies. The RL measurements on nanocrystal dispersions in water were carried out at room temperature on using a homemade apparatus featuring, as detection system, a charge coupled device (CCD) (Jobin-Yvon Spectrum One 3000) coupled to a spectrograph operating in the 200–1100 nm range (Jobin-Yvon Triax 180). The data were corrected for the spectral response of the detection system. RL excitation was obtained by X-rays irradiation through a Be window, using a Philips 2274 X-ray tube with tungsten target operated at 20 kV.

Immunofluorescence. C2C12 mouse myoblast cell line (ATCC® CRL-1772TM) was thawed and plated on cell culture treated dish in DMEM high glucose (Gibco) supplemented with 15% fetal bovine serum (FBS, EuroClone), 1% Penicillin/Streptomycin (Gibco), 1% Fungizone (Gibco). For immunofluorescence staining, cells were trypsinized after 48 hours and re-plated onto 12 multi wells plates at a density of 5×10^4 cells/well. Before seeding, sterile coverslips were plated into each wells in order to grow the cells on a surface conceived for confocal microscopy. 24 hours later, Eu:HfO_2 nanocrystals were added directly to the cell culture medium at different concentrations: 0.1,0.25, 0.5,

1 and 5 mg/ml. Eu:HfO_2 nanocrystal uptake was accomplished in 24 hours and cells were then processed for immunofluorescence. C2C12 were washed with 1X PBS and fixed in 4% formaldehyde in PBS at 37°C for 4 minutes, followed by permeabilization with 0.1% Triton-X-100 in PBS for 10 min at room temperature. After washing, cells were incubated for 40 minutes with FITC-conjugated phalloidin (Sigma-Aldrich; St. Louis, MO) and 4'-6-diamidino-2-phenylindol (DAPI, Thermo Fisher Scientific) for F-actin and nuclei staining, respectively. Labelled C2C12 were observed with a Leica SP2 confocal microscope and RGB images of DAPI/Eu:HfO_2/phalloidin cells were acquired with a 60x (1.4 NA) immersion oil objective.

MTT test. For proliferation experiments, C2C12 cells were seeded in a 96 multi well plate at a density of 3×10^3/well. After 24 hours from seeding, Eu:HfO_2 nanocrystals were added at the different concentrations to cell media. MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma) assay was performed 24, 48, 72 ad 96 hours from Eu:HfO_2 nanocrystal cell labelling, according to manufacturer's instructions. Briefly, 50 μg/ml MTT solution was added to each wells and incubated for 4 hours at 37°C. The medium was then removed and 100 μl dimethylsulfoxide (DMSO, Sigma) was added to samples. Absorbance was measured at 560 nm (Glomax Discover, Promega). Unlabelled cells were used as controls. The assay was performed in triplicate for each condition.

ROS and Trypan tests. Evaluation of ROS released in vitro was determined 24, 48, 72 and 96 hours after Eu:HfO_2 nanocrystal labelling. C2C12 cells were seeded and labelled as described for the MTT test. For ROS assay we evaluated only the 0.5-mg/ml condition, since it was shown to guarantee high cytocompatibility and improved Eu:HfO_2 nanocrystal fluorescence labelling efficiency. ROS-GloTM H_2O_2 Assay (Promega) was used following the producer's Non-Lytic protocol in order to proceed with additional evaluation on the same cells, and relative luminescence units were measured by microplate reader (Glomax Discover, Promega). Unlabelled C2C12 cells were used as controls. Menadione treated cells (5×10^{-5} M, Sigma) were considered as positive control, since menadione is a metabolic accelerator inducing intense toxic oxidative stress.[54] ROS assay was followed by Trypan

Blue (Sigma) Exclusion Test for determining and counting the number viable cells, which has been used to normalize the results on the ROS concentration obtained for direct comparison between different cell culture samples and therefore calculate the data reported in Fig. 4.[55]

2. Time resolved photoluminescence (PL) data analysis.

The time resolved PL spectra reported in Fig. 2d and 4b show a complex behavior. The signal decay has been reproduced with an analytically multi-exponential function

$$I_{PL} \propto \sum_{1}^{i} A_i e^{-\frac{t}{\tau_i}} \qquad \text{Eq. S1}$$

The parameters used for the fitting procedure are reported below in Tabs. 1 - 3 for Eu:HfO_2 in water, PBS and included in cells, respectively. The PL lifetime $<\tau>$ has been calculated as the weighted average of the characteristic decay time for each exponential function *i* using

$$<\tau> = \frac{\sum_{1}^{i} A_i \tau_i}{\sum_{1}^{i} A_i} \qquad \text{Eq. S2}$$

Table 1. Fit parameters for the PL decay at 613 nm of Eu:HfO_2 nanocrystals in H_2O.

	Fit Parameter	*Std Error*
y0	3.345e-2	3.715e-4
A_1	0.26	0.05
τ_1	177 µs	10 µs
A_2	0.74	0.05
τ_2	1170 µs	10 µs

Table 2. Fit parameters for the PL decay at 613 nm of Eu:HfO_2 nanocrystals in PBS.

	Fit Parameter	*Std Error*
y_0	3.535e-2	2.563e-4
A_1	0.11	0.02
τ_1	35 µs	11 µs
A_2	0.65	0.01

τ_2	455 μs	12 μs
A_3	0.24	0.01
τ_3	5584 μs	123 μs

Table 3. Fit parameters for the PL decay at 613 nm of Eu:HfO_2 nanocrystals included in cells.

	Fit Parameter	*Std Error*
y_0	7.910e-2	4.233e-3
A_1	0.23	0.06
τ_1	75 μs	30 μs
A_2	0.57	0.06
τ_2	455 μs	46 μs
A_3	0.20	0.01
τ_3	8084 μs	602 μs

3. Relative QY Measurements.

PL quantum yield measurements for Eu:HfO_2 dispersions have been conducted with an integrating sphere and by using a relative method referring to a fluorescence standard.[1] The nanocrystals QY under CW illumination at 532 nm was determined relative to the fluorescence QY_{std} = 0.68 of the standard dye Rhodamine B in ethanol according to the following equation

$$QY_{unk} = QY_{std}\left(\frac{As_{std}}{As_{unk}}\right)\left(\frac{I_{unk}}{I_{std}}\right)\left(\frac{P_{std}}{P_{unk}}\right)\left(\frac{\eta_{unk}}{\eta_{std}}\right)^2, \qquad \text{Eq. S3}$$

where QY_{unk}, As_{unk}, I_{unk}, P_{unk} and η_{unk} represent the quantum yield, absorptance at the excitation intensity, integrated photoluminescence spectral profile, excitation power density, and refractive index of the investigated material. The corresponding terms for the subscript "*std*" are for the reference quantum counter Rhodamine B in ethanol at the identical excitation wavelength.

4. Calculation of Nanocrystals Concentration.

The concentration of nanocrystals dispersed in water was determined by drying and calcining (in order to decompose organics) a known volume of dispersion. The so obtained mass of inorganic material was divided

by the initial dispersion volume in order to obtain the particle concentration (here expressed as mg/ml). The molarity of nanocrystals was calculated assuming the particles to be spheres with a diameter of 3.0 nm.

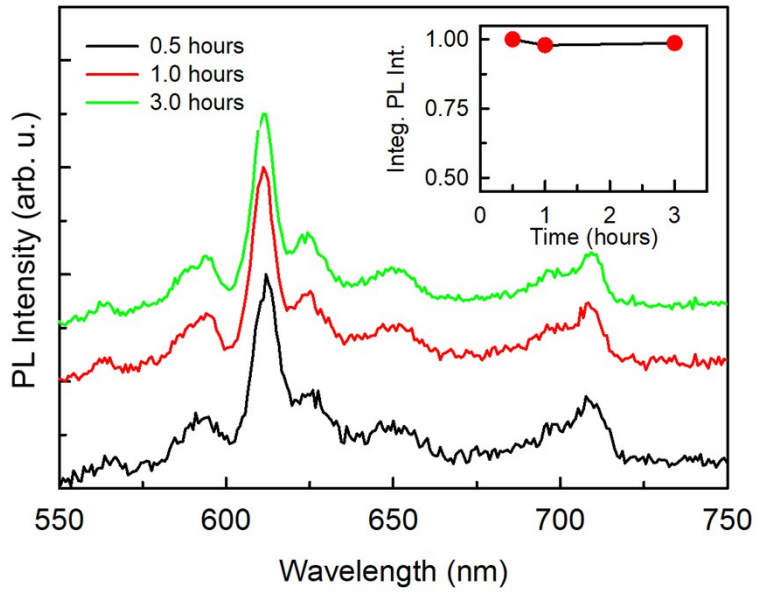

Fig. S1 PL spectrum of Eu:HfO_2 under laser excitation at 532 nm of fixed C2C12 cells stained Eu:HfO_2 nanocrystals (0.5 mg/ml) recorded at different times during continuous excitation. The inset reports the integrated PL signal, showing that after 3 hours of illumination there is no sign of significant degradation of the emitters embedded in cells.

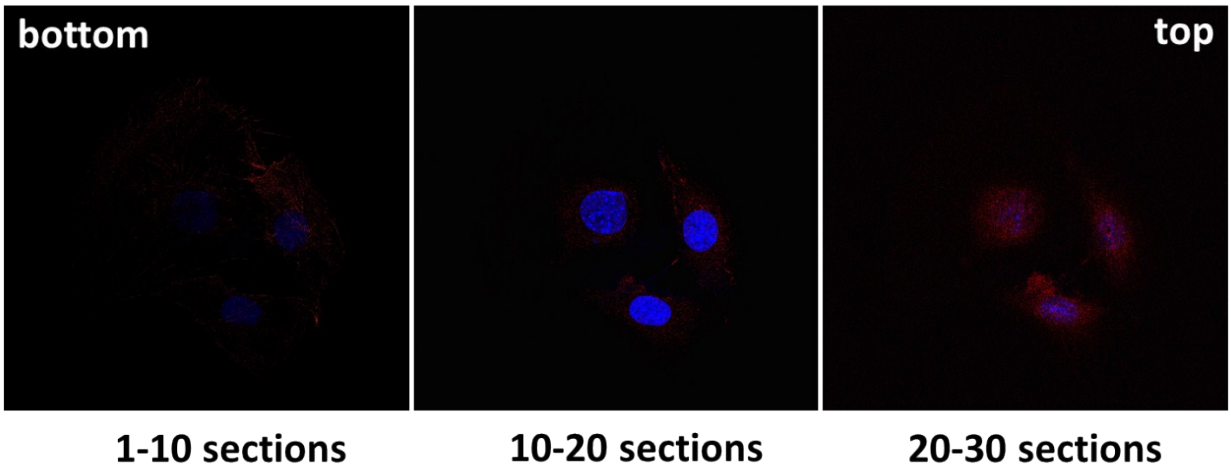

Fig. S2 Confocal fluorescence imaging of paraformaldehyde fixed C2C12 cells stained with DAPI (blue channel, specific for nuclei) and Eu:HfO_2 nanocrystals (red channel, 0.5 mg/ml). Each image is a z-stack of 10 transversal sections.

References

1. Montalti, M.; Credi, A.; Prodi, L.; Gandolfi, M. T., *Handbook of Photochemistry*. 3rd ed.; Taylor&Francis: Boca Raton, 2006.